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Suppression of Intestinal Neoplasia by DNA Hypomethylation

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Summary

We have used a combination of genetics and pharmacology to assess the effects of reduced DNA methyltransferase activity on *Apc*^{Min}-induced intestinal neoplasia in mice. A reduction in the DNA methyltransferase activity in *Min* mice due to heterozygosity of the DNA methyltransferase gene, in conjunction with a weekly dose of the DNA methyltransferase inhibitor 5-aza-deoxycytidine, reduced the average number of intestinal adenomas from 113 in the control mice to only 2 polyps in the treated heterozygotes. Hence, DNA methyltransferase activity contributes substantially to tumor development in this mouse model of intestinal neoplasia. Our results argue against an oncogenic effect of DNA hypomethylation. Moreover, they are consistent with a role for DNA methyltransferase in the generation of the C to T transitions seen at high frequency in human colorectal tumors.

Introduction

Methylation of mammalian DNA is found as the covalent modification of the fifth carbon position of the pyrimidine ring of cytosines in CpG dinucleotides (Adams and Burdon, 1982; Gardiner-Garden and Frommer, 1987; Antequera and Bird, 1993). Newly replicated DNA lacks this methylation in the nascent strand. Shortly after passage of the replication fork, a maintenance DNA methyltransferase methylates CpG dinucleotides on the newly synthesized strand at sites opposite to those methylated in the parental strand, thereby recreating the spectrum of methyl groups that existed prior to replication. So far, only one mammalian DNA (cytosine-5) methyltransferase enzyme (EC 2.1.1.37) has been identified and characterized (Bestor et al., 1988; Leonhardt and Bestor, 1993). The carboxy-terminal part of this protein shares sequence similarity with the conserved catalytic domain of prokaryotic DNA (cytosine-5) methyltransferases (Lauster et al., 1989; Posfai et al., 1989; Kumar et al., 1994). The mammalian enzyme has an additional large amino-terminal extension lacking sequence similarity to other known DNA methyltransferases. This amino-terminal region is thought to be a regulatory domain and is known to contain signals necessary for the targeted localization of the enzyme to replication foci in the nucleus (Leonhardt et al., 1992).

Changes in the pattern of DNA methylation have been correlated with a number of different processes in mammals. These include the expression level of genes (Yeivin and Razin, 1993), chromatin structure (Keshet et al., 1986; Tazi and Bird, 1990; Kass et al., 1993), the timing of DNA replication (Selig et al., 1988), genomic imprinting (Barlow, 1993; Li et al., 1993), and somatic X-chromosomal inactivation in females (Monk and Grant, 1990; Singer-Sam and Riggs, 1993). Whether DNA methylation is a cause or a consequence of these processes remains in large part to be resolved. Nevertheless, it is clear that DNA methylation is an essential process in mammalian development, since mouse embryos deficient for the known DNA methyltransferase do not survive past mid gestation (Li et al., 1992).

Several observations implicate a role for DNA methylation in cancer pathogenesis. Changes in the pattern of DNA methylation are commonly seen in human tumors (Jones and Buckley, 1990; Baylin et al., 1991; Spruck et al., 1993; Laird and Jaenisch, 1994). Some loci tend to show increased levels of DNA methylation (Ohtani-Fujita et al., 1993; Issa et al., 1994), whereas others are often hypomethylated (Feinberg and Vogelstein, 1983a, 1983b; Goelz et al., 1985; Rao et al., 1989; Vorce and Goodman, 1991; Hanada et al., 1993). The global level of DNA methylation is generally lower in tumor cells than in normal cells (Gama-Sosa et al., 1983; Feinberg et al., 1988). This decrease in the overall level of DNA methylation is curious in light of the normal to high levels of DNA methyltransferase expression usually seen in tumor cells (Kautiainen and Jones, 1986; El-Deiry et al., 1991; Issa et al., 1993).

Some investigators have proposed that changes in DNA methylation contribute to oncogenesis by affecting the expression levels of proto-oncogenes and tumor-suppressor genes. Thus, DNA hypomethylation would allow increased expression of oncogenes; alternatively, DNA hypermethylation would help to silence tumor-suppressor genes. However, there is no direct evidence that either mechanism is operative in oncogenesis. Indeed, it is not even clear whether the changes in DNA methylation play a causal role in oncogenesis or whether they are merely a consequence of the transformed state of the tumor cell.

We have designed experiments in an attempt to distinguish between these alternatives. Using a combination of genetics and pharmacology, we have manipulated the levels of functional DNA methyltransferase in mice and determined the consequences for early steps of the neoplastic process. To do so, we used mice heterozygous for the multiple intestinal neoplasia (*Min*) mutation of the adenomatosis polyposis coli (*Apc*) gene (*Min* mice) as a model system to assess the role of DNA methylation in early neoplastic transformation (Moser et al., 1990; Su et al., 1992; Luongo et al., 1993, 1994). *Min* mice develop

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multiple intestinal adenomas within the first few months of life (Moser et al., 1990, 1992; Luongo et al., 1994). The number of intestinal polyps present in these mice at specific ages offered us a readily quantifiable measure of any effects that changes in DNA methylation might have on the incidence or rate of polyp formation.

Min mice carry a germline point mutation in the *Apc* tumor-suppressor gene, the mouse homolog of the human *APC* gene, which, when present in mutant form in the human germ line, results in the syndrome of familial adenomatous polyposis coli (FAP; Su et al., 1992). FAP patients develop as many as 1000 benign colorectal polyps, some of which progress to malignancy if they are not removed (Groden et al., 1991; Joslyn et al., 1991; Ichii et al., 1992; Miyoshi et al., 1992; Powell et al., 1992; Nakamura, 1993). Similarly, mice carrying the *Min* allele of the *Apc* gene develop 100 or more intestinal polyps in the first 6 months of life. These polyps thus provide a model system for the early stages of human colorectal cancer, for which changes in DNA methylation patterns and in methyltransferase expression have been well documented (Gama-Sosa et al., 1983; Feinberg et al., 1988; Silverman et al., 1989; El-Deiry et al., 1991; Issa et al., 1993).

We have previously generated mice carrying germline mutations in the DNA methyltransferase gene (*Dnmt*; Li et al., 1992, 1993). Mouse embryos homozygous for either the $Dnmt^N$ allele (Li et al., 1992) or the stronger $Dnmt^S$ allele (Li et al., 1993) die before birth. Heterozygous mice have approximately half wild-type levels of DNA methyltransferase expression, which is apparently sufficient to maintain normal levels of DNA methylation (Li et al., 1992). They appear otherwise phenotypically indistinguishable from their wild-type littermates.

In the present study, we further perturbed methyltransferase activity through use of the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-aza-dC), which is known to titrate out the enzyme activity by a covalent trapping mechanism (Santi et al., 1983, 1984; Jüttermann et al., 1994). The combination of *Dnmt* heterozygosity and treatment with 5-aza-dC might be predicted to reduce the functional levels of DNA methyltransferase to below the threshold needed for maintenance of normal DNA methylation patterns. In this paper, we employ this combined strategy to study the effects of reduced DNA methyltransferase activity and consequent DNA methylation levels on the number of intestinal adenomas appearing in mice carrying the *Min* allele of the *Apc* gene.

Results

Effects of Decreased DNA Methyltransferase Activity on the Number of Intestinal Adenomas

To study the effects of DNA methyltransferase levels on intestinal polyp formation, we crossed 129/Sv $Dnmt^{S/+}$ female mice with C57BL/6 $Apc^{Min/+}$ males. In addition, we determined the maximal dose of 5-aza-dC that mice could tolerate during an extended treatment protocol starting at 1 week of age. This dose (5 μg of 5-aza-dC per 5 grams body weight) was given weekly by subcutaneous injection to the progeny of the cross described above. Control offspring were injected with phosphate-buffered saline (PBS) instead of 5-aza-dC. The injections were performed by investigators blind to the genotypes of the animals.

Previous experience indicated that at 100 days of age, a subset of intestinal polyps are just at the threshold of detectability in this strain background. During the subsequent 80 days, such polyps grow to a size that is readily visible. For this reason, mice were injected with either 5-aza-dC or PBS for 100 days (14 injections). The drug treatment was then discontinued, and the number of polyps was monitored 80 days later, allowing us to gauge the effects of DNA methyltransferase levels on polyp initiation and early growth during the first 100 days of life. In doing so, we avoided any cytotoxic or cytostatic effects that 5-aza-dC might have on the expansion of cell populations in the polyps during the growth period from 100 to 180 days. Figure 1 shows growth curves for the four different genotypes generated in this cross. As is apparent, the treatment with 5-aza-dC did not have a significant effect on the body weights of the mice.

A parallel cohort of animals was sacrificed at 100 days to determine the DNA methylation levels in the colon immediately following 5-aza-dC treatment. Figure 2 shows an analysis of DNA methylation levels of genomic colonic DNA derived from the *Dnmt* heterozygous and wild-type animals treated with or without 5-aza-dC. We compared DNA methylation levels by Southern blot analysis of re-

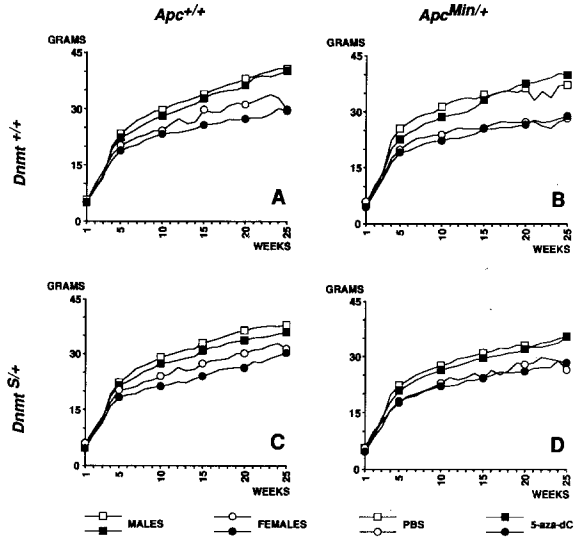

Figure 1. Growth Curves of Mice Treated Weekly with either PBS or 5-aza-dC

129/Sv $Dnmt^{S/+}$ female mice were crossed to C57BL/6 $Apc^{Min/+}$ males. Ungenotyped progeny were weighed and injected weekly with either 5 μg of 5-aza-dC per 5 g of bodyweight or with its solvent, PBS. The injections were continued for 100 days (14 injections), after which the mice were monitored for an additional 80 days and then sacrificed. The average weights were calculated for each of the genotypes, sexes, and treatments. The weight measurements were plotted by week. However, the symbols are indicated only every 5 weeks to preserve clarity. Squares, males; circles, females; open symbols, PBS treatment; closed symbols, 5-aza-dC treatment. (A) $Dnmt^{+/+}$, $Apc^{+/+}$ mice; (B) $Dnmt^{+/+}$, $Apc^{Min/+}$ mice; (C) $Dnmt^{S/+}$, $Apc^{+/+}$ mice; (D) $Dnmt^{S/+}$, $Apc^{Min/+}$ mice.

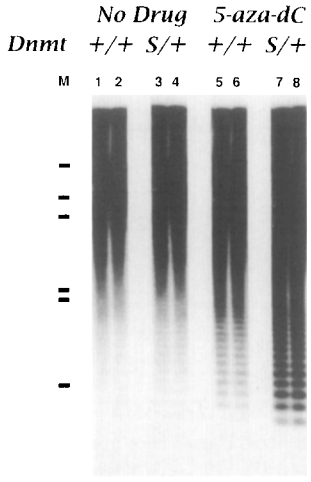

Figure 2. DNA Methylation Analysis of Genomic Colonic DNA from Mice Treated Weekly with or without 5-aza-dC

Mice derived from a cross between 129/Sv *Dnmt*$^{S/+}$ females and a C57BL/6 male were injected weekly with 5-aza-dC for 100 days (14 weeks) or untreated. Genomic DNA from colons collected from 100-day-old mice was isolated as described previously (Laird et al., 1991). Analysis of DNA methylation levels involved digestion of genomic DNA by the methylation-sensitive restriction enzyme HpaII, followed by hybridization with a centromeric minor satellite repeat probe (Chapman et al., 1984) for a reflection of the global DNA methylation status. The *Dnmt* genotypes and treatments are indicated above the lanes.

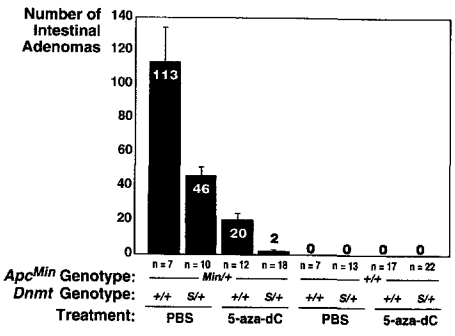

Figure 3. The Number of Intestinal Polyps in Mice Treated Weekly with either PBS or 5-aza-dC

129/Sv *Dnmt*$^{S/+}$ female mice were crossed to C57BL/6 *Apc*$^{Min/+}$ males. Ungenotyped progeny were weighed and injected weekly with either 5 μg of 5-aza-dC per 5 g of bodyweight or with its solvent, PBS. The injections were continued for 100 days (14 injections), after which the mice were monitored for an additional 80 days and then sacrificed. The entire intestine was excised immediately following sacrifice, fixed and washed with Bouin's fixative, and subjected to a systematic microscopic screen for polyp formation along the entire length of the intestine. The investigator counting the polyps was blind to both *Apc* and *Dnmt* genotypes. The average number of polyps for each group is shown as a bar with the average also indicated above or within the bar; SEM is indicated by the thin error bars. The treatments, genotypes of the mice, and number of mice in each experimental group are indicated below the bars. All mice for this experiment were generated by the same limited number of parents and were maintained in the same room with the same food and cage conditions.

striction digests with the DNA methylation–sensitive enzyme HpaII, hybridized with a centromeric minor satellite repeat probe (Chapman et al., 1984) (see Figure 2). In this assay, reduced levels of DNA methylation are revealed by a relative increase in lower molecular weight bands. The analysis in Figure 2 shows that both *Dnmt* heterozygosity and 5-aza-dC treatment have a synergistically hypomethylating effect on colonic DNA. The effect of 5-aza-dC appears to be stronger than that of *Dnmt* heterozygosity.

The number of intestinal polyps at sacrifice at 180 days was determined by examination of the mucosa over the entire length of the intestine under a dissecting microscope. Figure 3 shows the averaged results obtained for the various experimental groups. Mice wild-type at the *Apc* locus did not develop intestinal polyps with any combination of *Dnmt* genotype or drug treatment. *Apc*$^{Min/+}$ heterozygous mice developed an average of 113 polyps in the *Dnmt*$^{+/+}$ PBS control group. *Dnmt*^{S} heterozygosity reduced the number to a statistically significant lower average of only 46 polyps ($p \leq 0.001$ in Student's t test). A more pronounced effect was seen in the *Apc*$^{Min/+}$ heterozygous mice treated with 5-aza-dC. Treatment of *Dnmt* wild-type mice with 5-aza-dC reduced the number of polyps from 113 to an average of 20. The combined effects of *Dnmt* heterozygosity and 5-aza-dC treatment resulted in only 2 intestinal adenomas.

The decrease in polyp number in mice having reduced levels of active DNA methyltransferase was opposite to the result expected for a model in which global DNA hypomethylation contributes to the neoplastic process in colorectal cancer. The effect of DNA methyltransferase on *Min*-induced intestinal neoplasia appeared to be predominantly on polyp number rather than size or morphology. The adenomas in the *Dnmt*$^{S/+}$ heterozygous mice appeared to be somewhat smaller, but this was not confirmed by a systematic morphometric analysis. We did not find any evidence for a difference in the state of differentiation or in the progression of the adenomas from the different experimental groups, as assessed by histological examination of the polyps in *Min* mice from all four cohorts.

Significantly, a substantial reduction in polyp number was achieved in the absence of 5-aza-dC treatment. A slight reduction in DNA methylation levels in untreated *Dnmt* heterozygous colonic DNA is also evident in Figure 2 (see lanes 3 and 4). Reduced DNA methylation and the decreased number of intestinal polyps, seen in the context of *Apc*$^{Min/+}$ heterozygosity, are the first examples of observed phenotypic differences between *Dnmt* wild-type mice and *Dnmt*$^{S/+}$ heterozygotes. Furthermore, these results demonstrate that *Dnmt* is a genetic modifier of the *Min* phenotype. It is the second such modifier to be described, the first being the AKR/J allele of the modifier of *Min*-1 (*Mom-1*; Moser et al., 1992; Dietrich et al., 1993); however, *Dnmt* and *Mom-1* are distinct loci. *Mom-1* maps to mouse chromosome 4 (Dietrich et al., 1993), whereas *Dnmt* maps to mouse chromosome 9 (Copeland et al., 1993).

Early Administration of 5-aza-dC Is Required for Its Effect on Polyp Number

Although treatment of *Min* mice with 5-aza-dC had a dra-

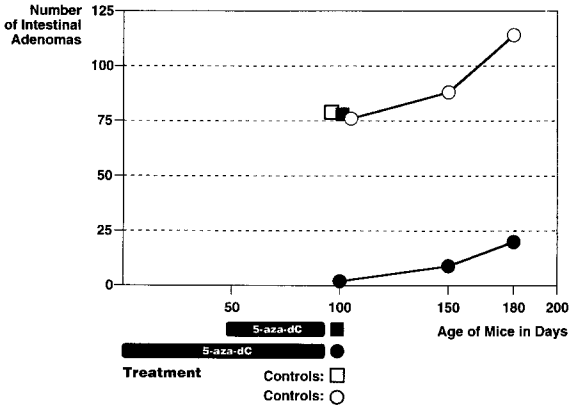

Figure 4. The Number of Intestinal Polyps in Mice with Various Timed Treatments and Time of Sacrifice

C57BL/6 $Apc^{Min/+}$ males were crossed to either 129/Sv (circles) or C57BL/6 (squares) female mice. The progeny were started on weekly injections of 5 μg of 5-aza-dC per 5 g of bodyweight at either 50 days (closed squares) or 7 days (closed circles) of age. Control mice (open symbols) were either injected with PBS or monitored without injection. In all cases, the injections were continued until the mice were 100 days old. The mice were sacrificed at either 100, 150, or 180 days of age, as indicated, and subjected to an intestinal polyp count as described for Figure 3. The sizes of the experimental groups were as follows: open squares, n = 6; closed squares, n = 9; open circles, n = 2, n = 5, and n = 13 for 100, 150, and 180 days, respectively; closed circles, n = 3, n = 2, and n = 12 for 100, 150, and 180 days, respectively. The treatments are indicated schematically at the bottom of the figure.

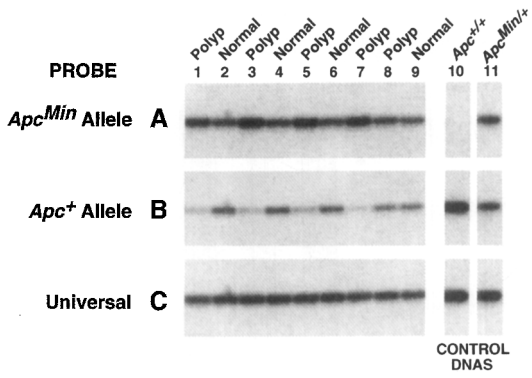

Figure 5. Analysis of Allelic Loss at the *Apc* Locus in Colon Adenomas

Intestines from randomly chosen mice from the experiment shown in Figure 3 were washed in PBS. Large, distinct adenomas were excised and processed for DNA isolation as described in Figure 2. In addition, normal intestinal mucosa adjacent to the polyp was excised and is shown immediately to the right of each polyp in the figure. The normal tissue in lane 9 is the control for both lanes 7 and 8. Lanes 10 and 11 contain samples derived from tail biopsies as control DNAs. Lane 10 is from an $Apc^{+/+}$ mouse. Lane 11 is from an $Apc^{Min/+}$ mouse. PCR reactions were performed as described in Experimental Procedures. The PCR products were gel-separated, blotted, and hybridized consecutively with oligodeoxynucleotide probes specific for the Apc^{Min} allele (A) and for the Apc^{+} allele (B), and finally with a probe recognizing both alleles (C).

matic effect on the number of intestinal polyps, the available evidence did not provide strong indications whether the effect was attributable to an early influence on the rate of initiation of adenoma formation or, alternatively, to a later cytostatic or cytotoxic influence of 5-aza-dC on already initiated adenomas. An indication that 5-aza-dC affects polyp initiation came from experiments in which drug treatment was delayed until day 50 in a cohort of C57BL/6 *Min* mice. In this strain background, the majority of polyps become visible between 50 and 100 days of age (A. F., unpublished data; Moser et al., 1990, 1992). As can be seen in Figure 4, the delay in the start of 5-aza-dC treatment abolished its inhibitory effect on the number of polyps counted at day 100. Furthermore, there was no readily apparent difference in the size of polyps from mice that had received a delayed 5-aza-dC treatment compared with their PBS-treated littermates. These results suggested that the initiation of polyp formation occurs prior to day 50 and is followed by a period of expansion of cell populations in already initiated polyps in the subsequent 50 day period. To exert its effects on polyp number, 5-aza-dC must be present during this critical formative period, rather than during the subsequent proliferative phase. As such, we consider it unlikely that 5-aza-dC acts through a cytotoxic or cytostatic effect on the cells in already formed adenomatous cell clones.

It is also apparent from Figure 4 that the majority of polyps at day 180 in mice treated from day 0 to 100 appeared only after cessation of drug treatment. These adenomas may represent rapidly growing polyps initiated after day 100, since the experiments described above argue against an inhibitory effect of 5-aza-dC on the expansion of previously initiated polyps. This raises the possibility that continued treatment with 5-aza-dC could lead to the virtual elimination of polyp formation.

Loss of Heterozygosity at the *Apc* Locus

The precise nature of the interactions of DNA methylation and the mutant Apc^{Min} allele in tumor development were not made clear by these analyses. The paradigm developed from extensive study of the *Apc* and similarly behaving tumor-suppressor genes dictates that loss of the wild-type *Apc* allele should accompany tumor development (Scrable et al., 1990; Weinberg, 1991; Boynton et al., 1992; Ichii et al., 1992; Levine, 1993; Nakamura, 1993; Brewster et al., 1994; Tamura et al., 1994). We wished to ascertain that this genetic mechanism operated in all of the experimental groups studied, regardless of the status of their *Dnmt* locus or their history of 5-aza-dC treatment.

We tested polyps from the different cohorts for loss of heterozygosity at the site of the Apc^{Min} mutation. A segment of DNA encompassing the mutation was amplified from DNA derived from polyps and from adjacent normal tissue. The polymerase chain reaction (PCR) products were hybridized consecutively with oligodeoxynucleotide probes specific for the Apc^{Min} allele and for the Apc^{+} allele, and finally with a probe recognizing both alleles. The relative strengths of the signals derived from the Apc^{Min}- and Apc^{+}-specific probes were calculated after quantitation on a phosphoimager and subsequent normalization to the signal deriving from the probe recognizing both alleles. Loss of heterozygosity in polyps should reveal a reduction

Table 1. Analysis of Allelic Loss at the *Apc* Locus in Intestinal Adenomas

Experimental Group	Number of Polyps Analyzed	Polyps with Loss of Heterozygosity	Polyps with No Loss of Heterozygosity
Wild-type control	12	11	1
Heterozygote control	6	5	1
Wild-type 5-aza-dC (0–100)	5	5	0
Wild-type 5-aza-dC (50–100)	4	3	1
Heterozygote 5-aza-dC (0–100)	5	4	1
Total	32	28	4

Intestines from randomly chosen mice from the experiment shown in Figure 1 and Figure 2 were washed in PBS. Large, distinct adenomas were excised and processed for DNA isolation as described previously (Laird et al., 1991). In addition, normal intestinal mucosa adjacent to the polyp was excised as a reference standard. PCR reactions were performed as described in Experimental Procedures. The PCR products were gel-separated, blotted, and hybridized consecutively with oligodeoxynucleotide probes specific for the Apc^{Min} allele and for the Apc^{+} allele, and finally with a probe recognizing both alleles. The relative hybridization signals were quantitated with a phosphoimager. Signals were first normalized using the hybridization signal specific for both alleles (Apc-Uni probe). Subsequently, the ratio of Apc^{Min} to Apc^{+} hybridization was calculated. Most polyps had ratios of more than 2.0. The four polyps defined as not showing loss of heterozygosity had ratios between 0.9 and 1.1, within the range seen for all normal adjacent tissues.

in the relative level of the Apc^{+} signal and an increase in the relative level of the Apc^{Min} signal in polyps compared with normal adjacent tissue.

Figure 5 shows the analysis of a representative series of polyps from *Dnmt* wild-type mice, along with control DNAs derived from tail biopsies from $Apc^{Min/+}$ and $Apc^{+/+}$ mice. The results obtained for all polyps analyzed are summarized in Table 1. Loss of the wild-type allele was seen in the majority of polyps derived from all experimental groups. These results are in agreement with the loss of heterozygosity at the *APC* locus seen as an early event in human cancer (Boynton et al., 1992; Ichii et al., 1992; Nakamura, 1993; Brewster et al., 1994; Tamura et al., 1994). The high frequency of loss of heterozygosity at the *Apc* locus is close to the 100% loss of *Apc* heterozygosity in Apc^{Min}-induced intestinal adenomas found by Luongo et al. (1994).

We found several polyps that lacked any sign of loss of heterozygosity (see Figure 5 and Table 1). Such cases do not seem to be overrepresented in any particular cohort. It is possible that these polyps have lost the function of the wild-type allele through another mechanism, such as the acquisition of a mutation elsewhere in the *Apc* gene. Alternatively, loss of function of the wild-type Apc protein may not be an absolute requirement for polyp formation. In support of this, we have recently obtained evidence for a dominant mode of action for the truncated protein product of the Apc^{Min} allele by comparing the phenotypes of mice heterozygous for different *Apc* mutations (A. F., unpublished data).

Discussion

DNA Methyltransferase Is an Oncogenic Determinant in *Min*-Induced Intestinal Neoplasia

Mice carrying the *Min* allele of the *Apc* gene are predisposed to intestinal polyps that become readily observable at several months of age (Moser et al., 1990, 1992; Luongo et al., 1994). As demonstrated here, the number of these polyps can be reduced in two ways: through treatment with the drug 5-aza-dC or through introduction of a mutant allele of the DNA methyltransferase gene. These two perturbants are also united by a common biochemical mechanism of action. When incorporated into DNA, 5-aza-dC causes the formation of stable covalent adducts between DNA methyltransferase and the DNA, resulting in the depletion of free, active enzyme from the cell (Santi et al., 1983, 1984; Jüttermann et al., 1994). The $Dnmt^{S}$ mutation achieves the same end result through inactivation of a chromosomal gene copy encoding this enzyme. Indeed, both *Dnmt* heterozygosity and 5-aza-dC treatment result in a reduction of genomic DNA methylation levels as represented by an analysis of centromeric repeat sequences.

The genesis of intestinal polyps depends upon the frequency with which they are initiated and their subsequent ability to proliferate to a size that makes them visible under the dissecting microscope. Thus, the present phenomena need to be interpreted in light of these two events governing observed polyp number. The initiation of polyp formation would appear to depend upon low frequency stochastic events, in that the number of distinct clones of polyps is many orders of magnitude lower than the number of cells in the target tissue. This stochastic event could involve the loss of the wild-type *Apc* allele that initially coexists with the mutant Apc^{Min} allele in all cells of the intestinal epithelium. While this is an attractive idea, our results suggest that there are events other than the loss of *Apc* heterozygosity that can be rate-limiting for polyp formation. This is apparent from the fact that the effects of DNA methyltransferase levels on polyp number do not seem to be mediated through a change in the rate of loss of *Apc* heterozygosity. If such were the case, then we should have found a decreased rate of loss of *Apc* heterozygosity in the mice with reduced numbers of polyps (Table 1). This should have shown up as a relative increase in the number of polyps without loss of *Apc* heterozygosity. Since this is not what we observed, we suggest that the effects of DNA methyltransferase levels are exerted through a rate-limiting process other than loss of *Apc* heterozygosity.

An early, rate-limiting stochastic event rather than a later, proliferative process would appear to be the target of inhibition by the 5-aza-dC that we have administered

to these mice. We conclude this from our observations of the comparative effects of this drug administered at weekly intervals beginning either at the first week of life or only at day 50. The first protocol involving early administration severely depresses polyp number; in contrast, delayed administration has no effects on polyp number or size. During days 50–100 of life, these polyps undergo a size proliferation that causes them to become visible in the dissecting microscope. Therefore, we conclude that 5-aza-dC has minimal effects on proliferation but profound effects on the earlier initiation, which must occur well before day 50.

Consistent with this conclusion is our recent work that documents the cytostatic/cytotoxic effects of 5-aza-dC treatment. We have found that these antiproliferative effects are not mediated through loss of DNA methylation per se, but rather through the formation of stable covalent complexes between the DNA methyltransferase enzyme and the incorporated cytosine analog (Santi et al., 1983, 1984; Jüttermann et al., 1994). Consequently, cells that have lower levels of DNA methyltransferase are less sensitive to growth inhibition by 5-aza-dC (Jüttermann et al., 1994). In the present study, we observed the exact opposite: the ability of 5-aza-dC to suppress polyp formation was stronger in mice with levels of DNA methyltransferase reduced owing to the *Dnmt*^{s} mutation.

DNA Methyltransferase Levels and Polyp Formation: Possible Mechanisms

Studies on DNA methylation changes in human cancer cells have presented a somewhat confusing picture. Hypotheses to explain the involvement of DNA methylation in cancer fall broadly into two classes. The first type proposes that changes in DNA methylation contribute to the oncogenic process through effects on gene expression. The other type argues that the major role of DNA methylation in cancer is mediated through the disproportionately high rate of mutation of methylated cytosine residues.

Among the models that address gene expression–mediated effects of DNA methylation in oncogenesis, two opposing mechanisms have been proposed. One model proposes that the commonly observed global DNA hypomethylation in human tumors is of selective advantage to the tumor cell through facilitated proto-oncogene expression (Feinberg and Vogelstein, 1983b; Rao et al., 1989; Vorce and Goodman, 1991; Hanada et al., 1993). In the alternative model, high expression of DNA methyltransferase is thought to drive hypermethylation of specific loci such as tumor-suppressor genes or genes specific to differentiated cell types (Kautiainen and Jones, 1986; Greger et al., 1989; Silverman et al., 1989; Baylin et al., 1991; El-Deiry et al., 1991; Issa et al., 1993, 1994; Ohtani-Fujita et al., 1993). This would allow outgrowth of undifferentiated neoplastic cells. The suppression of polyp formation by the reduction of DNA methyltransferase and DNA methylation levels is inconsistent with the first mechanism, which favors an oncogenic effect of DNA hypomethylation. Our results do lend support for the tumor-suppressor gene hypermethylation model, although one would have to argue that reduction of global DNA methyltransferase levels would affect local DNA hypermethylation. It is not clear how regional DNA hypermethylation is achieved in the face of global DNA hypomethylation.

In contrast to the gene expression–mediated models discussed above, the mutation-mediated models propose that DNA methylation contributes to oncogenesis through a disproportionately high mutation rate of 5-methylcytosine residues (Jones et al., 1992; Spruck et al., 1993; Laird and Jaenisch, 1994). Deamination at cytosine-4 of both methylated and unmethylated cytosine residues is known to occur spontaneously to yield thymine and uracil, respectively (Wang et al., 1982; Ehrlich et al., 1986; Frederico et al., 1990; Shen et al., 1994). A uracil residue is ultimately substituted by thymine if it is not repaired prior to DNA replication. The occurrence of C to T transition mutations is considerably higher for 5-methylcytosine than for unmethylated cytosine residues. In fact, mutations of this sort are of significance in human tumorigenesis. Approximately 47% of the characterized point mutations in the p53 tumor-suppressor gene in colorectal tumors are nucleotide transitions within CpG dinucleotides, the target for DNA methylation (Greenblatt et al., 1994). This frequency contrasts with the fact that CpG dinucleotides occur within the coding region of the wild-type human p53 gene at a frequency of 3.3% (39 out of 1179). The target size for mutagenesis at either nucleotide within CpG dinucleotides is therefore 6.6%. Mutations at CpG dinucleotides are thus overrepresented approximately 7-fold in human colorectal tumors (Greenblatt et al., 1994). Reduced cellular DNA methylation levels could lead to a lower rate of spontaneous C to T transitions. If rate-limiting mutational events, in addition to the loss of the wild-type *Apc* allele, were required for polyp formation, then a reduced rate of C to T transitions could result in a lower number of intestinal polyps.

The high mutation rate of methylated cytosine residues might also be achieved through a different mechanism that directly involves the DNA methyltransferase enzyme (reviewed in Laird and Jaenisch, 1994). The covalent enzyme adduct formed between DNA methyltransferase and its substrate cytosine has been found to destabilize the amine group of the latter, resulting in an enhanced rate of deamination. Normally, this adduct is formed only ephemerally; the presence of the methyl donor S-adenosyl methionine (SAM) results in the rapid completion of the reaction and the disappearance of this destabilized intermediate. But, in the absence of sufficient amounts of SAM, the half-life of this intermediate may be substantially extended, resulting, in turn, in a great increase in cytosine deamination. This has been demonstrated experimentally for a prokaryotic DNA methyltransferase (Shen et al., 1992). If such a mechanism were also applicable to the mammalian enzyme, limiting levels of SAM in the intestinal epithelium could result in a mutagenic process with an activity correlating with DNA methyltransferase levels. We note in passing that diets deficient in methionine have been reported to predispose humans to increased numbers of colonic polyps (Giovannucci et al., 1993). Whether this is only an adventitious association or derives from a

mechanism associated with DNA methyltransferase–mediated deamination and mutation is not revealed by presently available evidence. Experiments in rodents have shown that methyl-deficient diets, which lead to SAM depletion, enhance liver tumorigenesis (Mikol et al., 1983; Shivapurkar et al., 1986).

Recently, the laboratories of Peter Jones and Richard Roberts have found evidence for yet another mechanism through which DNA methyltransferase enzymes could exert an effect on cytosine mutation rates (Yang et al., personal communication; Klimasauskas and Roberts, personal communication). Both groups have demonstrated that a prokaryotic DNA methyltransferase is capable of high affinity binding to GU mismatches. In addition, the enzyme is then capable of converting the uracil residue to thymine by the transfer of a methyl group. GU mismatch repair would be inhibited directly by the binding of the DNA methyltransferase as well as indirectly by the conversion of U to T, which is less efficiently repaired in a mismatch with G (Shenoy et al., 1987; Brown and Brown, 1989). Whether the mammalian DNA methyltransferase exhibits similar properties remains to be seen.

Experiments currently under way are designed to examine the precise effects of 5-aza-dC treatment and the inactivated *Dnmt* allele on the mutational rate of genes within specific tissues of the mouse. Such experiments should indicate whether these perturbants directly suppress mutation rate, and in turn have profound effects on the rate of polyp initiation in *Min* mice.

Experimental Procedures

Mice

Mice were maintained in the facilities of the Whitehead Institute for Biomedical Research and were fed ad libitum. Their diet consisted of Agway Prolab Rat, Mouse, and Hamster 3000 chow, which has a crude protein content of at least 22%, a crude fat content of at least 5%, and a crude fiber content of at most 5%. C57BL/6 $Apc^{Min/+}$ mice were purchased from the Jackson Laboratories, Bar Harbor, Maine. 129/Sv $Dnmt^{S/+}$ mice were generated in our facilities (Li et al., 1992, 1993).

5-aza-dC Injections

129/Sv $Dnmt^{S/+}$ female mice were crossed to C57BL/6 $Apc^{Min/+}$ males. Progeny were weighed on a Mettler balance and injected weekly with either 5 μg of 5-aza-dC per 5 g of body weight (rounded to the nearest multiple of 5 g) or with its solvent, PBS. The 5-aza-dC (Sigma Chemical Co., St. Louis, MO, catalog number A-3656) was prepared as a 2.5 mg/ml solution in PBS and stored in aliquots at −70°C. Injections were performed with a 100 μl Hamilton 700 series syringe with a Luer tip and a PB600 dispenser attachment, allowing injection of 50 discrete 2 μl volumes. The injections were started at 7 days of age and continued for 100 days (14 injections), after which the mice were monitored for an additional 80 days and then sacrificed.

Polyp Analysis

The entire intestine was excised immediately following sacrifice, fixed, and washed with Bouin's fixative and subjected to a systematic microscopic screen for polyp formation along the entire length of the intestine. Adenomas were counted if they had reached a size of at least two villi. The investigator counting the adenomas was blind to both *Apc* and *Dnmt* genotypes. Adenomas destined for DNA analysis were microdissected from intestines that had been washed with PBS, before being fixed with Bouin's fixative.

DNA Methylation Analysis

DNA was prepared from frozen tissue samples as described previously (Laird et al., 1991). Genomic DNA (5 μg) was digested with the restriction enzyme HpaII (New England Biolabs, Beverly, MA) as specified by the manufacturer. After agarose gel electrophoresis followed by Southern blotting, the filters were hybridized with a 150 bp probe containing centromeric minor satellite repeat sequences derived from plasmid pMR150 (Chapman et al., 1984).

Allelic Loss Analysis

The murine *Apc* cDNA sequence was obtained from GenBank (accession no. M88127; Su et al., 1992). A 619 bp fragment spanning the site of the *Min* mutation was amplified from genomic DNA samples by PCR using oligonucleotides MAPC-15 (*Apc* sequence 2859–2841; 5′-TTCCACTTTGGCATAAGGC-3′) and MAPC-9 (*APC* sequence 2241–2258; 5′-GCCATCCCTTCACGTTAG-3′). The PCR products were gel-fractionated on a 2% agarose gel, Southern blotted, and hybridized with three probes in sequential hybridizations. The three probes were Min-Sense (*Apc* sequence 2542–2556; 5′-AGAAGTTAGGAGAGA-3′), Apc-Sense (*Apc* sequence 2542–2556; 5′-AGAAGTTTGGAGAGA-3′), and Apc-Uni (*Apc* sequence 2530–2544; 5′-TCTGAGAAAGACAGA-3′). Min-Sense hybridizes specifically to the Apc^{Min} mutant allele, while Apc-Sense hybridizes specifically to the wild-type allele. Apc-Uni hybridizes to both alleles. The hybridization signals were quantitated using a Fuji phosphoimager. The signal levels in the Apc-Uni hybridization were used to normalize the amount of DNA in each lane.

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References

Adams, R. L., and Burdon, R. H. (1982). DNA methylation in eukaryotes. CRC Crit. Rev. Biochem. *13*, 349–384.

Antequera, F., and Bird, A. (1993). CpG islands. In DNA Methylation: Molecular Biology and Biological Significance, J. P. Jost and H. P. Saluz, eds. (Basel, Switzerland: Birkhauser Verlag), pp. 169–185.

Barlow, D. P. (1993). Methylation and imprinting: from host defense to gene regulation? Science *260*, 309–310.

Baylin, S. B., Makos, M., Wu, J. J., Yen, R. W., de Bustros, A., Vertino, P., and Nelkin, B. D. (1991). Abnormal patterns of DNA methylation in human neoplasia: potential consequences for tumor progression. Cancer Cells *3*, 383–390.

Bestor, T., Laudano, A., Mattaliano, R., and Ingram, V. (1988). Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases. J. Mol. Biol. *203*, 971–983.

Boynton, R. F., Blount, P. L., Yin, J., Brown, V. L., Huang, Y., Tong, Y., McDaniel, T., Newkirk, C., Resau, J. H., Raskind, W. H., et al. (1992). Loss of heterozygosity involving the *APC* and *MCC* genetic loci occurs in the majority of human esophageal cancers. Proc. Natl. Acad. Sci. USA *89*, 3385–3388.

Brewster, S. F., Browne, S., and Brown, K. W. (1994). Somatic allelic loss at the DCC, APC, nm23-H1 and p53 tumor suppressor gene loci in human prostatic carcinoma. J. Urol. *151*, 1073–1077.

Brown, T. C., and Brown, L. M. (1989). G/U lesions are efficiently corrected to G/C in SV40 DNA. Mutat. Res. *227*, 233–236.

Chapman, V., Forrester, L., Sanford, J., Hastie, N., and Rossant, J. (1984). Cell lineage-specific undermethylation of mouse repetitive DNA. Nature *307*, 284–286.

Copeland, N. G., Jenkins, N. A., Gilbert, D. J., Eppig, J. T., Maltais, L. J., Miller, J. C., Dietrich, W. F., Weaver, A., Lincoln, S. E., Steen, R. G., Stein, L. D., Nadeau, J., and Lander, E. S. (1993). A genetic linkage map of the mouse: current applications and future prospects. Science *262*, 57–66.

Dietrich, W. F., Lander, E. S., Smith, J. S., Moser, A. R., Gould, K. A., Luongo, C., Borenstein, N., and Dove, W. (1993). Genetic identification of *Mom-1*, a major modifier locus affecting *Min*-induced intestinal neoplasia in the mouse. Cell *75*, 631–639.

Ehrlich, M., Norris, K. F., Wang, R. Y., Kuo, K. C., and Gehrke, C. W. (1986). DNA cytosine methylation and heat-induced deamination. Biosci. Rep. *6*, 387–393.

El-Deiry, W. S., Nelkin, B. D., Celano, P., Yen, R. W., Falco, J. P., Hamilton, S. R., and Baylin, S. B. (1991). High expression of the DNA methyltransferase gene characterizes human neoplastic cells and progression stages of colon cancer. Proc. Natl. Acad. Sci. USA *88*, 3470–3474.

Feinberg, A. P., and Vogelstein, B. (1983a). Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature *301*, 89–92.

Feinberg, A. P., and Vogelstein, B. (1983b). Hypomethylation of ras oncogenes in primary human cancers. Biochem. Biophys. Res. Commun. *111*, 47–54.

Feinberg, A. P., Gehrke, C. W., Kuo, K. C., and Ehrlich, M. (1988). Reduced genomic 5-methylcytosine content in human colonic neoplasia. Cancer Res. *48*, 1159–1161.

Frederico, L. A., Kunkel, T. A., and Shaw, B. R. (1990). A sensitive genetic assay for the detection of cytosine deamination: determination of rate constants and the activation energy. Biochemistry *29*, 2532–2537.

Gama-Sosa, M. A., Slagel, V. A., Trewyn, R. W., Oxenhandler, R., Kuo, K. C., Gehrke, C. W., and Ehrlich, M. (1983). The 5-methylcytosine content of DNA from human tumors. Nucl. Acids Res. *11*, 6883–6894.

Gardiner-Garden, M., and Frommer, M. (1987). CpG islands in vertebrate genomes. J. Mol. Biol. *196*, 261–282.

Giovannucci, E., Stampfer, M. J., Colditz, G. A., Rimm, E. B., Trichopoulos, D., Rosner, B. A., Speizer, F. E., and Willett, W. C. (1993). Folate, methionine, and alcohol intake and risk of colorectal adenoma. J. Natl. Cancer Inst. *85*, 875–884.

Goelz, S. E., Vogelstein, B., Hamilton, S. R., and Feinberg, A. P. (1985). Hypomethylation of DNA from benign and malignant human colon neoplasms. Science *228*, 187–190.

Greenblatt, M. S., Bennett, W. P., Hollstein, M., and Harris, C. C. (1994). Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res. *54*, 4855–4878.

Greger, V., Passarge, E., Hopping, W., Messmer, E., and Horsthemke, B. (1989). Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. Hum. Genet. *83*, 155–158.

Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M., et al. (1991). Identification and characterization of the familial adenomatous polyposis coli gene. Cell *66*, 589–600.

Hanada, M., Delia, D., Aiello, A., Stadtmauer, E., and Reed, J. C. (1993). bcl-2 gene hypomethylation and high-level expression in B-cell chronic lymphocytic leukemia. Blood *82*, 1820–1828.

Ichii, S., Horii, A., Nakatsuru, S., Furuyama, J., Utsunomiya, J., and Nakamura, Y. (1992). Inactivation of both APC alleles in an early stage of colon adenomas in a patient with familial adenomatous polyposis (FAP). Hum. Mol. Genet. *1*, 387–390.

Issa, J. P., Vertino, P. M., Wu, J., Sazawal, S., Celano, P., Nelkin, B. D., Hamilton, S. R., and Baylin, S. B. (1993). Increased cytosine DNA-methyltransferase activity during colon cancer progression. J. Natl. Cancer Inst. *85*, 1235–1240.

Issa, J.-P., Ottaviano, Y. L., Celano, P., Hamilton, S. R., Davidson, N. E., and Baylin, S. B. (1994). Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. Nature Genet. *7*, 536–540.

Jones, P. A., and Buckley, J. D. (1990). The role of DNA methylation in cancer. Adv. Cancer Res. *54*, 1–23.

Jones, P. A., Rideout, W. M., Shen, J. C., Spruck, C. H., and Tsai, Y. C. (1992). Methylation, mutation and cancer. Bioessays *14*, 33–36.

Joslyn, G., Carlson, M., Thliveris, A., Albertsen, H., Gelbert, L., Samowitz, W., Groden, J., Stevens, J., Spirio, L., Robertson, M., et al. (1991). Identification of deletion mutations and three new genes at the familial polyposis locus. Cell *66*, 601–613.

Jüttermann, R., Li, E., and Jaenisch, R. (1994). Toxicity of 5-aza-2′-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. Proc. Natl. Acad. Sci. USA *91*, 11797–11801.

Kass, S. U., Goddard, J. P., and Adams, R. L. (1993). Inactive chromatin spreads from a focus of methylation. Mol. Cell. Biol. *13*, 7372–7379.

Kautiainen, T. L., and Jones, P. A. (1986). DNA methyltransferase levels in tumorigenic and nontumorigenic cells in culture. J. Biol. Chem. *261*, 1594–1598.

Keshet, I., Lieman, H. J., and Cedar, H. (1986). DNA methylation affects the formation of active chromatin. Cell *44*, 535–543.

Kumar, S., Cheng, X., Klimasauskas, S., Mi, S., Posfai, J., Roberts, R. J., and Wilson, G. G. (1994). The DNA (cytosine-5) methyltransferases. Nucl. Acids Res. *22*, 1–10.

Laird, P. W., and Jaenisch, R. (1994). DNA methylation and cancer. Hum. Mol. Genet. *3*, 1487–1495.

Laird, P. W., Zijderveld, A., Linders, K., Rudnicki, M. A., Jaenisch, R., and Berns, A. (1991). Simplified mammalian DNA isolation procedure. Nucl. Acids Res. *19*, 4293.

Lauster, R., Trautner, T. A., and Noyer-Weidner, M. (1989). Cytosine-specific type II DNA methyltransferases. A conserved enzyme core with variable target-recognizing domains. J. Mol. Biol. *206*, 305–312.

Leonhardt, H., and Bestor, T. H. (1993). Structure, function and regulation of mammalian DNA methyltransferase. In DNA Methylation: Molecular Biology and Biological Significance, J. P. Jost and H. P. Saluz, eds. (Basel, Switzerland: Birkhauser Verlag), pp. 109–119.

Leonhardt, H., Page, A. W., Weier, H. U., and Bestor, T. H. (1992). A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. Cell *71*, 865–873.

Levine, A. J. (1993). The tumor suppressor genes. Annu. Rev. Biochem. *62*, 623–651.

Li, E., Bestor, T. H., and Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell *69*, 915–926.

Li, E., Beard, C., and Jaenisch, R. (1993). Role for DNA methylation in genomic imprinting. Nature *366*, 362–365.

Luongo, C., Gould, K. A., Su, L. K., Kinzler, K. W., Vogelstein, B., Dietrich, W., Lander, E. S., and Moser, A. R. (1993). Mapping of multiple intestinal neoplasia (*Min*) to proximal chromosome 18 of the mouse. Genomics *15*, 3–8.

Luongo, C., Moser, A. R., Gledhill, S., and Dove, W. F. (1994). Loss of Apc^+ in intestinal adenomas from *Min* mice. Cancer Res. *54*, 5947–5952.

Mikol, Y. B., Hoover, K. L., Creasia, D., and Poirier, L. A. (1983). Hepatocarcinogenesis in rats fed methyl-deficient, amino acid-defined diets. Carcinogenesis *4*, 1619–1629.

Miyoshi, Y., Ando, H., Nagase, H., Nishisho, I., Horii, A., Miki, Y., Mori, T., Utsunomiya, J., Baba, S., Petersen, G., et al. (1992). Germ-line mutations of the *APC* gene in 53 familial adenomatous polyposis patients. Proc. Natl. Acad. Sci. USA *89*, 4452–4456.

Monk, M., and Grant, M. (1990). Preferential X-chromosome inactiva-

tion, DNA methylation and imprinting. Development (Suppl.), 55–62.

Moser, A. R., Pitot, H. C., and Dove, W. F. (1990). A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science *247*, 322–324.

Moser, A. R., Dove, W. F., Roth, K. A., and Gordon, J. I. (1992). The *Min* (multiple intestinal neoplasia) mutation: its effect on gut epithelial cell differentiation and interaction with a modifier system. J. Cell Biol. *116*, 1517–1526.

Nakamura, Y. (1993). The role of the adenomatous polyposis coli (*APC*) gene in human cancers. Adv. Cancer Res. *62*, 65–87.

Ohtani-Fujita, N., Fujita, T., Aoike, A., Osifchin, N. E., Robbins, P. D., and Sakai, T. (1993). CpG methylation inactivates the promoter activity of the human retinoblastoma tumor-suppressor gene. Oncogene *8*, 1063–1067.

Posfai, J., Bhagwat, A. S., Posfai, G., and Roberts, R. J. (1989). Predictive motifs derived from cytosine methyltransferases. Nucl. Acids Res. *17*, 2421–2435.

Powell, S. M., Zilz, N., Beazer-Barclay, Y., Bryan, T. M., Hamilton, S. R., Thibodeau, S. N., Vogelstein, B., and Kinzler, K. W. (1992). *APC* mutations occur early during colorectal tumorigenesis. Nature *359*, 235–237.

Rao, P. M., Antony, A., Rajalakshmi, S., and Sarma, D. S. (1989). Studies on hypomethylation of liver DNA during early stages of chemical carcinogenesis in rat liver. Carcinogenesis *10*, 933–937.

Santi, D. V., Garrett, C. E., and Barr, P. J. (1983). On the mechanism of inhibition of DNA-cytosine methyltransferases by cytosine analogs. Cell *33*, 9–10.

Santi, D. V., Norment, A., and Garrett, C. E. (1984). Covalent bond formation between a DNA-cytosine methyltransferase and DNA containing 5-azacytosine. Proc. Natl. Acad. Sci. USA *81*, 6993–6997.

Scrable, H. J., Sapienza, C., and Cavenee, W. K. (1990). Genetic and epigenetic losses of heterozygosity in cancer predisposition and progression. Adv. Cancer Res. *54*, 25–62.

Selig, S., Ariel, M., Goitein, R., Marcus, M., and Cedar, H. (1988). Regulation of mouse satellite DNA replication time. EMBO J. *7*, 419–426.

Shen, J. C., Rideout, W. M., and Jones, P. A. (1992). High frequency mutagenesis by a DNA methyltransferase. Cell *71*, 1073–1080.

Shen, J.-C., Rideout, W. M., and Jones, P. A. (1994). The rate of hydrolytic deamination of 5-methylcytosine in double-stranded DNA. Nucl. Acids Res. *22*, 972–976.

Shenoy, S., Ehrlich, K. C., and Ehrlich, M. (1987). Repair of thymine.guanine and uracil.guanine mismatched base-pairs in bacteriophage M13mp18 DNA heteroduplexes. J. Mol. Biol. *197*, 617–626.

Shivapurkar, N., Wilson, M. J., Hoover, K. L., Mikol, Y. B., Creasia, D., and Poirier, L. A. (1986). Hepatic DNA methylation and liver tumor formation in male C3H mice fed methionine- and choline-deficient diets. J. Natl. Cancer Inst. *77*, 213–217.

Silverman, A. L., Park, J. G., Hamilton, S. R., Gazdar, A. F., Luk, G. D., and Baylin, S. B. (1989). Abnormal methylation of the calcitonin gene in human colonic neoplasms. Cancer Res. *49*, 3468–3473.

Singer-Sam, J., and Riggs, A. D. (1993). X chromosome inactivation and DNA methylation. In DNA Methylation: Molecular Biology and Biological Significance, J. P. Jost and H. P. Saluz, eds. (Basel, Switzerland: Birkhauser Verlag), pp. 358–384.

Spruck, C. H., Rideout, W. M., and Jones, P. A. (1993). DNA methylation and cancer. In DNA Methylation: Molecular Biology and Biological Significance, J. P. Jost and H. P. Saluz, eds. (Basel, Switzerland: Birkhauser Verlag), pp. 487–509.

Su, L. K., Kinzler, K. W., Vogelstein, B., Preisinger, A. C., Moser, A. R., Luongo, C., Gould, K. A., and Dove, W. F. (1992). Multiple intestinal neoplasia caused by a mutation in the murine homolog of the *APC* gene. Science *256*, 668–670. Erratum: Science *256*, 1114.

Tamura, G., Maesawa, C., Suzuki, Y., Tamada, H., Satoh, M., Ogasawara, S., Kashiwaba, M., and Satodate, R. (1994). Mutations of the *APC* gene occur during early stages of gastric adenoma development. Cancer Res. *54*, 1149–1151.

Tazi, J., and Bird, A. (1990). Alternative chromatin structure at CpG islands. Cell *60*, 909–920.

Vorce, R. L., and Goodman, J. I. (1991). Hypomethylation of ras oncogenes in chemically induced and spontaneous B6C3F1 mouse liver tumors. J. Toxicol. Environ. Health *34*, 367–384.

Wang, R. Y., Kuo, K. C., Gehrke, C. W., Huang, L. H., and Ehrlich, M. (1982). Heat- and alkali-induced deamination of 5-methylcytosine and cytosine residues in DNA. Biochim. Biophys. Acta *697*, 371–377.

Weinberg, R. A. (1991). Tumor suppressor genes. Science *254*, 1138–1146.

Yeivin, A., and Razin, A. (1993). Gene methylation patterns and expression. In DNA Methylation: Molecular Biology and Biological Significance, J. P. Jost and H. P. Saluz, eds. (Basel, Switzerland: Birkhauser Verlag), pp. 523–568.

Note Added in Proof

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